Characterizing Mucous Cell Remodeling in Cystic Fibrosis
Relationship to Neutrophils

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Rationale: **Relatively few studies have characterized mucous cells or mucins in detail in cystic fibrosis (CF), and the relationship between mucous cell abnormalities and neutrophilic inflammation is uncertain.**

Objectives: **To characterize mucous cell phenotypes and mucin profiles in CF and to determine if neutrophils accumulate around goblet cells in the epithelium and gland acini in the submucosa.**

Methods: **Bronchial biopsies were collected from 7 subjects with CF and 15 control subjects, and the morphology of mucous cells was measured. Immunostains for gel-forming mucins and neutrophil elastase were quantified.**

Measurements and Main Results: **Goblet cell size was increased in CF ($p = 0.004$), but the number of goblet cells was normal. The volume of submucosal glands was fourfold higher than normal ($p = 0.031$), but the proportion of mucous and serous cells in CF glands was normal. The patterns of expression of gel-forming mucins in epithelial and submucosal compartments in CF were similar to normal. Although neutrophil elastase immunostaining was intense in the epithelium in CF, neutrophils were largely absent around gland acini in the submucosa.**

Conclusion: **The most prominent pathologic feature in the CF airway is an increase in submucosal gland volume, but serous cell transdifferentiation to mucous cells does not occur, nor are gland acini inflamed with neutrophils. The mechanism for increased submucosal gland volume in CF deserves further study.**

Keywords: cystic fibrosis; MUC5AC; MUC5B; neutrophil elastase; submucosal glands

The pathologic hallmarks of airway disease in cystic fibrosis (CF) include inflammation, especially neutrophilic inflammation, and airway remodeling, especially remodeling of mucous cells. Neutrophilic airway inflammation is one of the earliest findings in CF, occurring in infants before the onset of obvious infection (1). Neutrophil products such as proteases are important, both as growth factors for mucous cells and as secretagogues for mucins. For example, neutrophil elastase stimulates mucin gene expression and mucin secretion in airway epithelial cells *in vitro* and *in vivo* (2–6) and induces airway mucous metaplasia in hamsters and mice (7, 8). These findings suggest that neutrophils may be found in close proximity to mucous cells in the epithelium or submucosa in CF, but little information exists about the relationship between neutrophilic inflammation and mucous cells in the epithelium and glands.

Remodeling of mucous cells in the surface epithelium and in submucosal glands is characteristic of CF, but surprisingly few studies have examined mucous cell phenotypes in CF in detail or have characterized the expression patterns of gel-forming mucins in the surface epithelium and submucosa. Morphologic studies of lungs from infants show that submucosal glands are normal in size and number (9, 10), regardless of the presence of airway infection (10). However, autopsy studies in older patients with CF show that submucosal glands are usually prominent (11), with a preponderance of mucous acini within the glands (12). Thus, patients with CF are not born with mucous cell abnormalities, but clear changes occur by the time of death. Data on the intervening years are limited, because very few studies have used bronchoscopy to investigate the airway phenotype of patients living with CF.

Gel-forming mucins, such as MUC2, MUC5AC, and MUC5B, are secreted by airway mucous cells, and these mucins are subject to regulation by inflammatory stimuli (13, 14). Changes in mucin gene expression are therefore likely to occur in CF, but most studies have been in the upper airway and have yielded conflicting results. One study shows decreased MUC5AC expression in nasal epithelial cells from patients with CF (15), whereas another shows increased MUC2 expression (16). Recently, mucin proteins have been quantified in airway secretions in CF. Surprisingly, it was found that MUC5B and MUC5AC concentrations are lower than normal (17). To our knowledge, there has been no study quantifying the expression of gel-forming mucins in tissues from the lower airway in CF.

In this study, we examined neutrophilic inflammation and mucous cell remodeling in CF by performing detailed analyses of neutrophils, mucous cells, and mucins in tissue sections from biopsies obtained during research bronchoscopy. We used stereology to quantify the size and number of mucous cells, and their mucin expression patterns. We also examined the spatial distribution of neutrophils around goblet cells in the surface epithelium and gland acini in the submucosa. Some of these data have been previously presented in abstract form (18).

METHODS

Subjects

Seven persons with CF and 15 healthy control subjects were enrolled. Subjects with CF were 23 to 37 years old, met Cystic Fibrosis Foundation criteria for the diagnosis of CF, had compatible lung disease, and sweat chloride testing results ranging from 82 to 120 mM/L. Five subjects were homozygous for ΔF508, one subject was ΔF508/3905insT, and one subject was 1717-1/unidentified. Five of the seven subjects with CF had sputum that was culture positive for *Pseudomonas aeruginosa* at the time of the study. Control subjects were healthy volunteers, aged 22 to 44, with PC_{20} methacholine greater than 16 mg/ml and FEV_1 greater than 80% predicted. Subjects were excluded for a CF exacerbation or

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TABLE 1. BASELINE CLINICAL CHARACTERISTICS OF THE HEALTHY AND CYSTIC FIBROSIS SUBJECTS

Characteristic	Healthy (*n* = 15)	Cystic Fibrosis (*n* = 7)
Age, yr	31 ± 7	28 ± 5
Sex, M:F	8:7	2:5
FEV_1, L	3.9 ± 0.66	2.1 ± 0.37*
FEV_1, % predicted	104.7 ± 12.3	59.7 ± 9.14*
FEV_1/FVC	0.81 ± 0.09	0.64 ± 0.11†

Definition of abbreviations: F = female; M = male.
Data are mean ± SD.
* $p < 0.0001$ versus healthy control subjects by *t* test.
† $p = 0.0009$ versus healthy control subjects by *t* test.

respiratory infection within the previous 6 wk, smoking history (> 10 pack-years lifetime or any cigarette smoking in the last year), history of hemoptysis requiring intensive care unit admission, or other CF complication that significantly increased the risk of bronchoscopy. All subjects provided written, informed consent and the University of California–San Francisco Committee on Human Research approved the study. Biopsy samples from these same subjects had previously been used for analysis of airway smooth muscle morphology (19, 20).

Protocol

At Visit 1, subjects were characterized with a medical history, physical examination, spirometry, allergen skin-prick testing, sputum induction, and methacholine challenge. At Visit 2 (1 wk later), the subjects underwent bronchoscopy with endobronchial biopsies using a specific method described previously and in accordance with guidelines for research bronchoscopy (20, 21). Additional methodologic details are described in the online supplement. Subjects with CF underwent a third visit 1 wk after bronchoscopy for spirometry and a physical examination.

Stereology

Design-based stereology (22) was used to quantify submucosal gland volume, epithelial mucin stores, goblet cell size, basal lamina thickness, epithelial height, and immunostaining. In the submucosal space, the volume of glands and volume of immunostains for MUC2, MUC5AC, MUC5B, neutrophil elastase, and transforming growth factor (TGF)-α were measured and referenced to total submucosal volume. In addition, the volume of serous and mucous cells in glands was measured and referenced to total gland volume. In the epithelium, the volume of stored mucin and volume of immunostains for MUC2, MUC5AC, MUC5B, neutrophil elastase, and TGF-α were measured and referenced to total epithelial volume and to the surface area of basal lamina. We quantified goblet cell volume and number using the rotator technique (23) (60× lens, 2,600× total magnification) on Alcian blue/periodic acid Schiff–stained sections. We measured basal lamina thickness and epithelial height using the orthogonal intercept method previously described (24). All stereologic measurements were performed in a blinded fashion using an integrated microscope (Olympus, Albertslund, Denmark), video camera (JVC Digital Color; JVC A/S, Tatstrup, Denmark), automated microscope stage, and computer (Dell Optiplex GS270 PC Running Computer-assisted Stereology Toolbox software; Olympus). Details involving the stereologic techniques as well as immunohistochemistry protocols for mucins, neutrophil elastase, and TGF-α are outlined in the online supplement.

Statistics

Two-group comparisons were performed using Student's *t* test. The Pearson correlation coefficient was used to relate the neutrophil elastase staining with the submucosal gland volume. Analyses were made using Prism (GraphPad, San Diego, CA) and a p value of less than 0.05 was considered significant (additional details in the online supplement).

RESULTS

Subjects

The clinical characteristics of the study subjects are shown in Table 1. Subjects with CF had moderate to severe airflow obstruction, with FEV_1 ranging from 45 to 71% predicted. Five of the seven subjects with CF (71%) were allergic to at least one aeroallergen, a fraction that was not different from healthy subjects (12 of the 15 healthy subjects, 80%). In addition, the mean number of positive skin tests in the healthy subgroup (2.86) and CF subgroup (2.42) was not significantly different ($p = 0.697$).

Epithelium and Goblet Cells

The epithelium was higher than normal in CF (Table 2). There was a trend for an increase in epithelial mucin stores in CF (as referenced to the surface area of basal lamina), and this was a consequence of an increase in the average size of goblet cells, rather than in the number of goblet cells (Table 2).

Submucosal Glands

The volume fraction of submucosal glands normalized to the surface area of basal lamina was fourfold greater in subjects with CF than in healthy subjects (Figure 1). The volume of serous cells as a fraction of overall gland volume was similar in CF and healthy subjects (Figure 2, Table 2); likewise, the volume of mucous cells as a fraction of overall gland volume was no different in CF and healthy subjects (Figure 2, Table 2). Thus, the increase in gland volume in CF is the result of increases in both serous and mucous cell compartments, with no change in the overall proportions of these gland cell types.

TABLE 2. MUCIN STORES, GOBLET CELL CHARACTERISTICS, AND MUCIN PROTEIN IMMUNOHISTOCHEMISTRY IN THE AIRWAY EPITHELIUM AND SUBMUCOSAL GLANDS OF HEALTHY SUBJECTS AND SUBJECTS WITH CYSTIC FIBROSIS

Outcome	Healthy, Mean (*n* = 15)	CF, Mean (*n* = 7)	Difference	95% CI	p Value
Epithelial height, μm	31.66	40.46	8.80	4.607 to 12.99	0.0003*
Volume of epithelial mucin per surface area of basal lamina, $\mu m^3/\mu m^2$	2.778	3.327	0.549	−0.890 to 1.987	0.436
Mean goblet cell volume, μm^3	2,239	3,312	1073	379.6 to 1767	0.004*
Number of goblet cells per surface area of basal lamina, no./mm^2	1,469	1,108	−360.8	−1,239 to 517	0.402
Volume of submucosal gland per volume of submucosa, mm^3/mm^3	0.039	0.108	0.069	0.005 to 0.133	0.036*
Volume of serous cell acini per volume of gland, mm^3/mm^3	0.376	0.412	0.060	−0.178 to 0.249	0.733
Volume of mucous cell acini per volume of gland, mm^3/mm^3	0.642	0.631	−0.012	−0.211 to 0.188	0.905
Volume of neutrophil elastase per volume of submucosa, mm^3/mm^3	0.027	0.021	−0.007	−0.025 to 0.011	0.414
Volume of neutrophil elastase per volume of epithelium, mm^3/mm^3	0.017	0.057	0.040	0.022 to 0.058	0.0002*
Volume of epithelial TGF-α per surface area of basal lamina, $\mu m^3/\mu m^2$	3.671	7.023	3.351	−1.100 to 7.803	0.132
Volume of submucosal TGF-α per surface area of basal lamina, $\mu m^3/\mu m^2$	0.438	1.282	0.844	0.201 to 1.487	0.013*

Definition of abbreviations: CI = confidence interval; CF = cystic fibrosis; TGF-α = transforming growth factor α.
* $p < 0.05$ versus healthy control subjects by *t*-test.

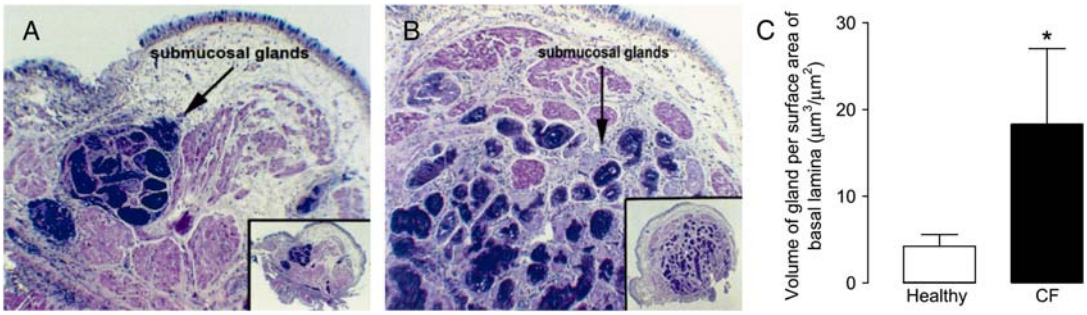

Figure 1. Submucosal gland volume in healthy subjects and subjects with cystic fibrosis (CF). Representative photomicrographs of endobronchial biopsies stained with periodic acid Schiff and Alcian blue photographed at 5× showing submucosal glands (*arrows*) in (*A*) a healthy subject and (*B*) a subject with CF. (*C*) Data are mean ± SEM and represent 15 healthy subjects and 7 subjects with CF. There is a fourfold increase in gland volume in subjects with CF. * Significantly different from healthy subjects; $p = 0.03$.

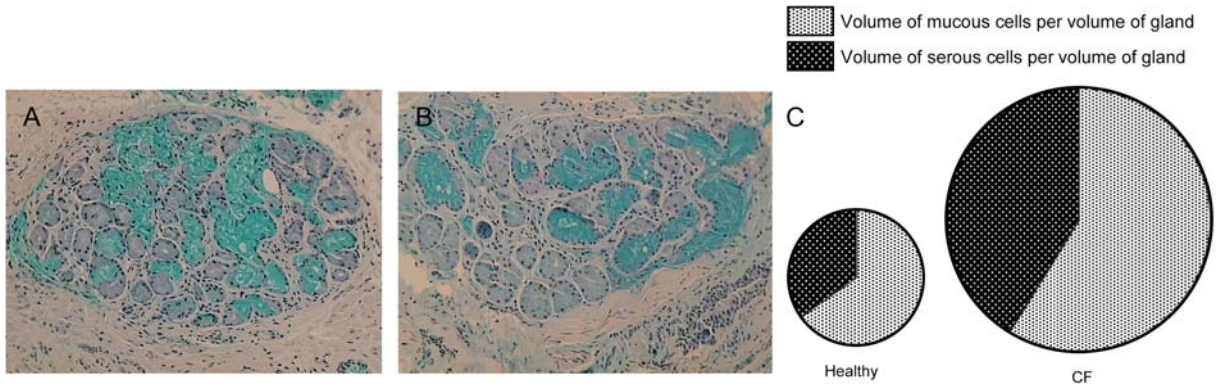

Figure 2. Mucous and serous cell volume in healthy subjects and subjects with CF. Representative photomicrographs of endobronchial biopsies stained with Alcian blue and photographed at 10× showing serous and mucous acini in (*A*) healthy subjects and (*B*) subjects with CF. (*C*) Data are means and represent 15 healthy subjects and 7 subjects with CF. The volume of serous cells per volume of gland was 0.38 in subjects with CF and 0.41 in healthy subjects. The volume of mucous cells per volume of gland was 0.63 in subjects with CF and 0.64 in healthy subjects. There was no significant difference in the volume of serous cells per volume of gland or volume of mucous cells per volume of gland between subjects with CF and healthy subjects.

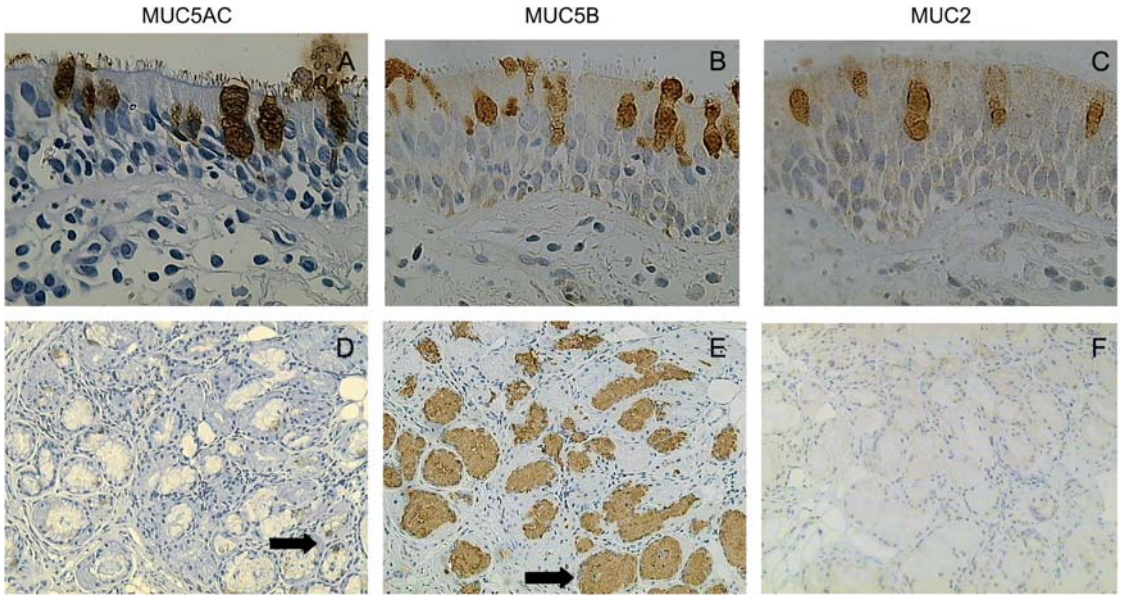

Figure 3. Immunostains of mucins. Photomicrographs of representative sections from bronchial biopsies from a patient with CF showing epithelium at 40× (*upper panels*) and submucosal glands at 10× (*lower panels*). Sections are stained with antibodies to (*A, D*) MUC5AC, (*B, E*) MUC5B, and (*C, F*) MUC2. *Arrows* display immunostain in mucous cells of submucosal glands. MUC5AC is the predominant mucin in the epithelium, whereas MUC5B is the predominant mucin in the glands.

Mucin Glycoproteins

In the surface epithelium, MUC5AC was the most prominent gel-forming mucin in both healthy and CF subjects, and we found no difference in MUC5AC between groups (Figures 3 and 4). In the submucosa, MUC5B immunostaining was very prominent in glands with little or no immunostaining for MUC2 or MUC5AC. The volume of MUC5B immunostain was higher than normal in the CF submucosa, but when normalized to the volume of glands, there was no difference between groups (Figure 4).

Neutrophil Elastase

Neutrophil elastase immunostaining was most intense in the epithelium of CF biopsies and, when quantified, the volume of neutrophil elastase staining in the epithelium was fourfold higher in subjects with CF than in healthy subjects (Figure 5). In contrast, neutrophil elastase immunostaining was minimal in the region of submucosal glands in CF (Figure 5). When quantified, the volume of neutrophil elastase staining in the submucosa was similar in subjects with CF and healthy subjects (Figure 5). In hematoxylin and eosin–stained sections from the same subjects, we found polymorphonuclear cells (PMNs) in the epithelial layer in CF and only rarely in the epithelial layer of healthy subjects (Figure E2). We did not find PMNs in the submucosal gland regions in these hematoxylin and eosin sections, consistent with the immunostaining results.

TGF-α Immunostaining

TGF-α is an important ligand for the epithelial growth factor receptor (EGFR), and EGFR signaling has been implicated in the mechanism of mucin gene up-regulation and epithelial tubulogenesis (25, 26). Thus, we examined the CF tissue sections for evidence of TGF-α expression. TGF-α staining, as detected by the MF9 antibody, was evident in epithelial cells in a perinuclear distribution (Figure 6), and the volume of immunostain was higher in the subjects with CF but not significantly so (Table 2). In the submucosa, TGF-α immunostain was very prominent in glands (Figure 6), and the volume of TGF-α was significantly greater than normal, reflecting the higher gland volume in CF (Table 2).

Epithelial Tubulogenesis

An increase in gland volume may result from new gland growth through a process of epithelial tubulogenesis, whereby glands form as a result of invaginations or buds of surface epithelial cells. We examined biopsy tissues from the subjects with CF for evidence of these buds or invaginations. Although not frequent, we did find structures in the CF sections close to the epithelium that could be new gland forming (Figure 7). This finding needs to be interpreted cautiously not only because of our limited

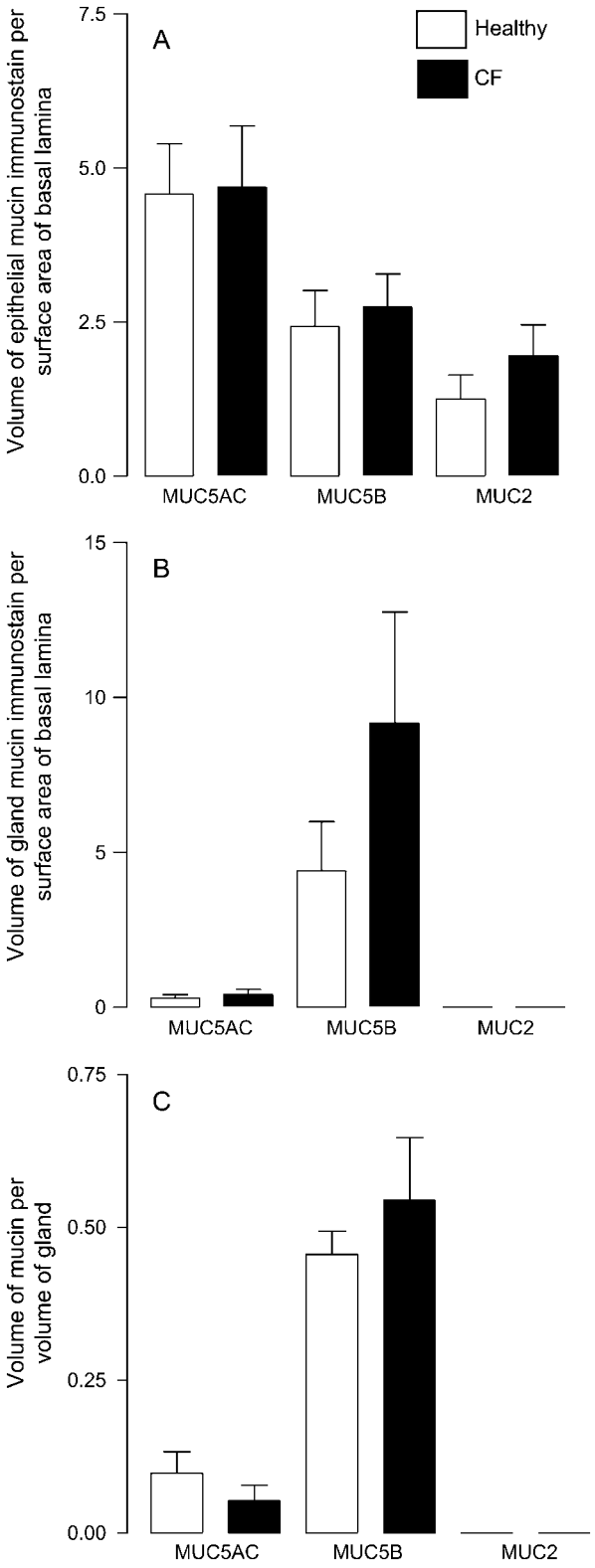

Figure 4. MUC5AC, MUC5B, and MUC2 immunostains in the epithelium, submucosa, and submucosal glands in healthy subjects and subjects with CF. In the epithelium, all subjects (15 healthy and 7 CF) displayed immunostaining for MUC5AC, MUC5B, and MUC2. In the submucosa, 9 of 15 healthy subjects and 5 of 7 subjects with CF had positive immunostaining for MUC5AC; 13 of 15 healthy subjects and 6 of 7 subjects with CF had positive immunostaining for MUC5B; none of the healthy subjects or subjects with CF had positive immunostaining for MUC2. Data are mean ± SEM. (*A*) There was no significant difference between healthy subjects and subjects with CF in the volume of epithelial MUC5AC, MUC5B, and MUC2 immunostain per surface area of basal lamina. (*B*) There was no significant difference between healthy subjects and subjects with CF in the volume of gland mucin immunostain per surface area of basal lamina. (*C*) There was no difference between healthy subjects and subjects with CF in the volume of mucin immunostain per volume of gland.

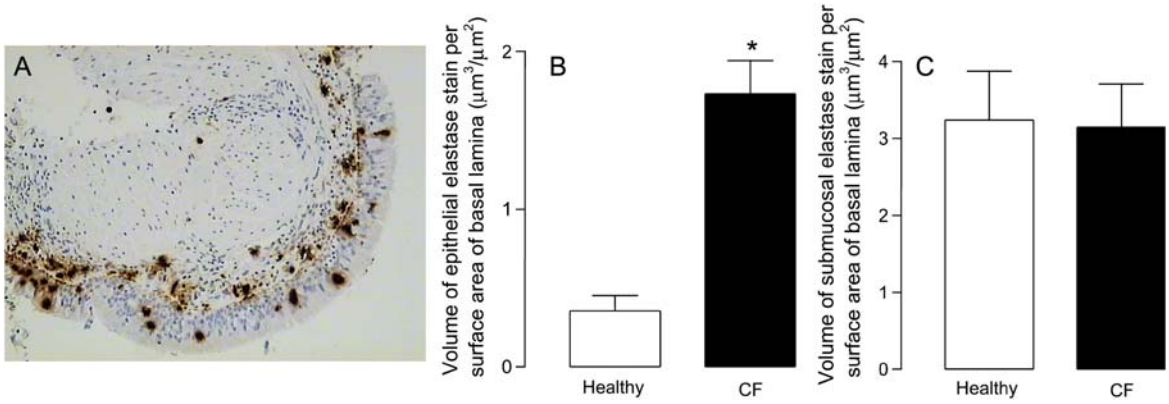

Figure 5. Neutrophil elastase immunostain in the epithelium and submucosa of healthy subjects and subjects with CF. (*A*) Representative photomicrograph of an endobronchial biopsy photographed at 5× from a subject with CF, immunostained with NP57. Neutrophil elastase staining is most prominent in the epithelial space and immediate subepithelial space. (*B*) Data are means ± SEM. There was a threefold increase in the volume of epithelial elastase immunostain in subjects with CF. (C) There is no significant difference in neutrophil elastase staining in the submucosal space between healthy subjects and subjects with CF. * Significantly different from healthy subject; $p < 0.0001$.

sample size but also because there are no markers that distinguish new glands from old. A related finding in the CF biopsy sections was that the basal lamina zone was 20% thinner than normal (Figure 8), a characteristic that may facilitate epithelial tubulogenesis.

Neutrophil Elastase and Mucous Cell Remodeling

We considered it possible that neutrophilic inflammation in the surface epithelium is mechanistically linked to remodeling of goblet cells and submucosal gland cells. When using data from all subjects, we found a direct relationship between the volume of neutrophil elastase immunostaining in the epithelial layer and the volume of glands in the submucosa ($r = 0.53$, $p = 0.01$). This correlation did not reach statistical significance when using the data for the subjects with CF alone because of the limited number of subjects.

DISCUSSION

Although there have been *post mortem* studies of the airway pathology of CF (10–12, 27), we believe ours is the first study to quantify mucous cells, mucins, and inflammatory changes in CF using tissue obtained by bronchoscopy and methods of design-based stereology. We found that the volume fraction of submucosal glands was fourfold higher than normal in CF, a finding that is much more striking than the changes in goblet cells, which were more subtle. These subtleties included a modest increase in the volume of stored mucin in the epithelium, which was the result of increased goblet cell size rather than increase in goblet cell number. Mucin stores in goblet cells may be relatively low in CF because of ongoing degranulation signals. Nevertheless, we were surprised that we did not find more evidence of goblet cell hyperplasia in the large airways in CF.

Using immunohistochemistry, we found that MUC5AC was the predominant mucin expressed by goblet cells, and MUC5B was the predominant mucin in gland mucous cells. This pattern was not different in subjects with CF compared with healthy subjects. Thus, although the large inflammatory stimuli in CF may up-regulate mucin gene expression in mucous cells in the epithelium and submucosa, they do not alter basic patterns of expression of gel-forming mucins. The increased mucin stores in the submucosal glands represent a reservoir of mucins that could contribute to mechanisms of mucin hypersecretion in CF. Although mechanisms of mucous cell degranulation are distinct from those of intracellular mucin accumulation, it is reasonable to assume that increases in mucin stores in the submucosa lead to increases in mucins in secretions.

Neutrophilic airway inflammation is a known pathologic feature of CF, but our goal was to examine neutrophils in relation to goblet cells in the epithelium and gland cells in the submucosa. We found that neutrophil elastase immunostaining was largely absent around glands in the submucosa, so that neutrophil-mediated degranulation of gland mucous cells is very unlikely.

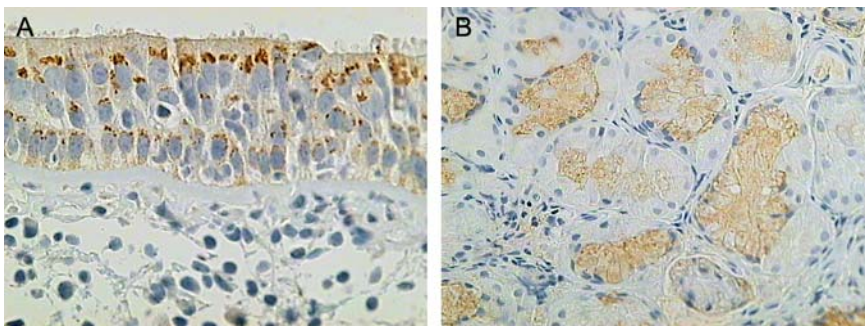

Figure 6. Transforming growth factor (TGF)-α immunostain in the epithelium and submucosal space of healthy subjects and subjects with CF. Photomicrographs of representative endobronchial biopsy tissue sections from a subject with CF, photographed at (*A*) 40×, showing TGF-α immunostaining to be most prominent in the perinuclear region of the epithelial cells, and at (*B*) 20×, showing TGF-α immunostaining in the submucosal glands.

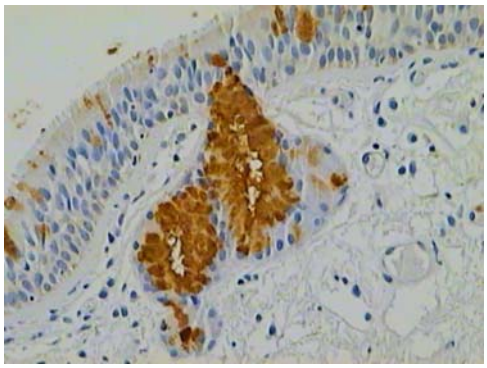

Figure 7. Photomicrograph of a bronchial biopsy tissue section from a patient with CF stained for MUC5B and counterstained with hematoxylin. A tuboacinar structure originating from the surface epithelium and with duct epithelium staining positive for MUC5B is seen. This structure has a short collecting duct and a simple bilobed acinar appearance that could represent a new gland forming.

Other mucin secretagogues must regulate gland mucin secretion in CF. In contrast, in the epithelium, we found intense neutrophil elastase immunostaining. It is unclear what causes neutrophils to accumulate in the epithelium. Recent data suggest that there are epithelial cell changes related to CFTR deficiency that cause neutrophil accumulation in the epithelial layer (28, 29). For example, epithelial cells in CF have an augmented production of interleukin 8 (30–32) and increased intercellular adhesion molecule-1 expression (28, 29). Whatever the mechanism of neutrophil accumulation in the epithelial layer, the consequences could include degranulation of goblet cells, or stimulation of goblet cell hypertrophy.

The increased volume of glands in the submucosa was the most striking aspect of the pathologic phenotype that we observed in the subjects with CF. Our data showing an increase in the volume fraction of glands in the submucosa could result from the numbers of gland units being increased, or because individual glands become hypertrophied. Our methods do not allow us to distinguish between these possibilities. We are able to state that the gland volume increases with proportionate increases in the serous and mucous acini. Published data do not shed light on whether gland enlargement in CF results from growth of new submucosal glands, the enlargement of existing glands, or both. There are data to support the concept of new gland growth in adult airways. For example, in health, submucosal glands are confined to large cartilaginous airways. However, in autopsy studies of CF lungs, submucosal glands are found in more distal, noncartilaginous airways (33). In addition, using animal models, it is possible to induce gland growth in the airways (34, (35), suggesting that new gland growth is possible in adult lungs. Any such development of glands from surface epithelial cells would require epithelial progenitor cells and recapitulation of the epithelial tubulogenesis that occurs during lung development. During fetal lung development, buds form from the surface epithelium followed by lateral morphogenesis into the mesenchyme (36–38), a sequence that can be reproduced in cell culture. Normal human tracheobronchial epithelial cells cocultured with human fetal lung fibroblasts penetrate collagen matrices (39), form tubular structures, and undergo dichotomous branching (40). Polarized noninvasive epithelial cells may transform into nonpolar invasive mesenchymal cells ("epithelial mesenchymal transition"), break through the basement membrane, and invade surrounding extracellular matrix (41).

Our study design did not address the mechanism of gland enlargement in CF, but we hypothesize that neutrophil-directed epithelial cell activation could lead to epithelial mesenchymal transition, epithelial tubulogenesis, and new glands. Our findings for the epithelial-restricted nature of neutrophilic inflammation in CF and the positive correlation between epithelial neutrophils and gland volume in the submucosa provide some support for this hypothesis as does the finding of thinning of the basal lamina zone in CF. This latter result could occur because of the actions of neutrophil proteases and thinning of the basal lamina, which could, in turn, facilitate tubulogenesis. Interactions between epithelial cells and extracellular matrix have been particularly well studied in the kidney. For example, locally produced growth factors, such as EGFR ligands, have roles in nephrogenesis and in renal regeneration after acute tubular injury (42, 43). Both the number and extent of tyrosine phosphorylation of EGF receptors increase in the developing kidney during late fetal development, coincident with the timing of tubulogenesis and glomerulogenesis (26, 43). TGF-α has been shown to be important for branching tubulogenesis of renal epithelial cells in matrigel, and in complete metanephric organ culture, in which induction of the mesenchyme by ureteric budding occurs. In this

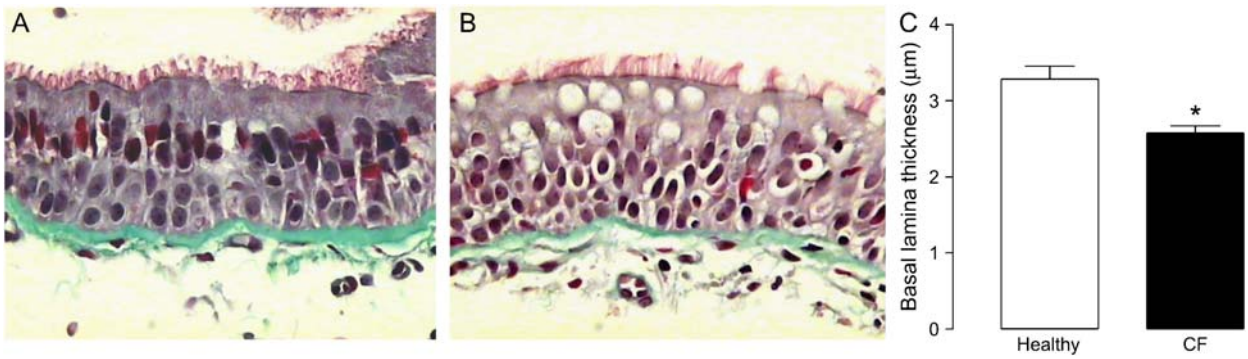

Figure 8. Thickness of the reticular basement membrane zone in healthy subjects and subjects with CF. Photomicrograph of trichrome-stained biopsy sections photographed at 40× demonstrate a thinner reticular basement membrane (RBM) zone in (*B*) subjects with CF compared with (*A*) healthy subjects. (C) Data are mean + SEM and represent 15 healthy subjects and 7 subjects with CF. * Significantly different from healthy subjects; $p = 0.0097$.

context, our finding that TGF-α expression in the submucosa in CF is restricted to submucosal glands suggests that this particular ligand for EGFR may play a role in the development and maintenance of gland structures in CF.

Conflict of Interest Statement: Neither author has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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